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(54) Title: SOLID PHASE ASSEMBLY AND RECONSTRUCTION OF BIOPOLYMERS

(57) Abstract

The present invention concerns the construction of solid phase assembly of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.

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SOLID PHASE ASSEMBLY AND RECONSTRUCTION OF BIOPOLYMERS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns the construction of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.

Background of the Invention

DNA is the chemical substance that makes up the genomes of most life forms. Two properties of DNA that are fundamental to its in vivo function and to the ability of scientists to manipulate it in vitr are that (i) DNA is composed of four different subunits ("bases"), adenine (A), guanine (G), cytosine (C) and thymine (T), linked together by a sugar-phosphate backbone to form long polymeric strands, and (ii) two "complementary" strands of DNA come together to form a double helical DNA molecule by specific hydrogen bonded base pairing (A pairs with T and G pairs with C). This specific base pairing plays an important role in chromosomal replication, a process in which the two DNA strands of a chromosome become separated, then a DNA polymerase enzyme uses each strand as a "template" to synthesize a complementary strand which then base pairs with the template strand, thereby resulting in the formation of two chromosomes from one.

Base pairing also functions in the process of "transcription," wherein an RNA polymerase enzyme utilizes the base pairing properties of one strand of a gene to synthesize a "messenger RNA" molecule (a nucleic acid in which uracil replaces thymine and the sugar is ribose instead of 2"-deoxyribose). The messenger RNA is subsequently "translated" into protein as directed by the genetic code (each 3-base '"codon" in the messenger RNA specifies a certain amino acid to be incorporated into a protein product). Thus, for each gene, the coupled transcription/translation process results in biosynthesis of a protein molecule that contains an amino acid sequence that is encoded by the base sequence in the DNA. In turn, the amino acid sequence in the protein determines how the protein folds into a specific structure and how it interacts with other molecules in its biochemical function, for example, catalysis of a specific chemical reaction in the case of an enzyme.

Finally, base pairing forms the basis of the "annealing" reaction that is employed in a variety of laboratory DNA manipulations: Two separated DNA strands will spontaneously pair up to form a duplex structure throughout the region(s) of complementarity if, and only if, they contain one or more stretches of complementary base sequence.

Recent development of techniques for manipulation of genes and proteins, which are used extensively in the fields of genetic engineering and biotechnology has allowed chemical synthesis of DNA molecules of any desired base sequence, which can be used to alter existing genes or create new ones. This capability, a fundamental tool in "protein engineering," allows analysis of the structure/function relationships within proteins and creation of superior enzymes and drugs in the biotechnology and pharmaceutical industries.

Over the past thirty years, methods for chemical synthesis of DNA have rapidly developed. Michelson and Todd first chemically synthesized a dinucleotide containing the correct phosphodiester linkage (Michelson and Todd, J. Chem. Soc.:2632-2638 (1955)). Khorana developed and used the phosphate triester method of DNA synthesis to produce a gene encoding transfer RNA (Agarwal, et al., Nature (Lond.), 227:27-40, (1970)). More efficient phosphate triester DNA synthesis procedures were subsequently developed (Letsinger and Ogilvie, J. Am Chem. Soc., 89:4801-4803, (1967); Narong et al., Meth. Enzymol., 65:610-620 (Letsinger et al., J. Am. Chem. Soc., 97:3278-3279 (1975); Beaucage and Caruthers, Tet. Lett., 22:1859-1862, (1981)). Efficient utilization of solid phase supports for chemical synthesis of DNA have been disclosed (Matteucci and Caruthers, J. Am. Chem. Soc., 103:3185-3191 (1981); Sproat and Bannwarth, Tet. Lett., 24:5771 -5774 (1983)).

The use of solid phase supports for chemical synthesis of DNA contributed most importantly to the ability to rapidly and efficiently synthesize DNA chemically, because the growing chain is covalently attached to an insoluble support, permitting reagents to be washed away between chemical steps, thus eliminating the need to purify the polynucleotide product after each addition of monomer. Furthermore, solid phase synthesis permits automation of the process, so that each base addition (via multistep reaction cycle) can be carried out in about ten minutes at room temperature (Smith, American Biotechnology Laboratory (Dec., 1983); Caruthers, Science, 230:281-285 (1955)).

It is now possible to construct a duplex DNA molecule encoding a protein or portion thereof, and use of the synthetic duplex fragment to construct a recombinant DNA which can be expressed in vivo to obtain a novel gene product. However, the widespread application of gene

synthesis has been hindered by: (i) the high cost of synthesis of all the oligonucleotides needed to assemble an average gene (typically $5,000 to $20,000); and (ii) the slow and labor intensive nature of gene assembly from synthetic oligonucleotides. Chemical synthesis of DNA currently produces polynucleotides up to 100-150 bases in length (and at the upper limits the yield is very low). The coding portion of the average gene, however, consists of 1000-base pairs. Thus, in order to assemble a gene, a series of overlapping, complementary oligonucleotides must be synthesized, then "annealed" together (i.e., mixed together and incubated under conditions that favor formation of the double helix between complementary sequences within the two strands). The duplex DNA, which contains strand interruptions at alternating positions along the two strands, is then converted to a contiguous duplex segment, by enzymatic ligation. Only then can the duplex DNA be cloned into a vector for subsequent analysis and expression (protein production). In practice, the correct assembly of a gene from a complex mixture of oligonucleotides is difficult to achieve in a single annealing step, due to formation of a variety of undesirable annealing products. A series of laborious purification and analytical steps must normally be carried out before the intact gene is isolated.

Solid phase procedures for chemical synthesis of peptides are frequently based on the protocol of Merrifield, which has been successfully used for synthesis of enzymatically active, 124-residue ribonuclease A (Gutte and Merrifield, J. Biol. Chem., 246:1922-1941 (1971)). This procedure uses polystyrene-divinylbenzene supports, t-butyloxy-carbonyl (tboc) amino group protection, and DCC-activated condensation with symmetric anhydride intermediates, and has been adapted for fully automated peptide synthesis. Another procedure for chemical synthesis of peptides (known as the "Fmoc" procedure)

utilizes a composite polyamide-Kieselguhr support (superior for continuous flow synthesis), together with fluorenylemethoxycarbonyl (Fmoc) amino group protection, and N-hydroxybenzatriazole-activated condensation with pentafluorophenyl ester (PFPE) intermediates or symmetrical anhydride intermediates (Auffret and Meade, Synthetic Peptides in Biology and Medicine, Alitalo et al. (Eds.), Elsevier Science Publishers, Amsterdam, (1985)).

As with DNA, chemical synthesis of peptides, prior to the present invention was possible for chain lengths up to 100-200 residues (with very low yields at these upper limits). More typically, peptides of 20-30 residues are produced. Assembly of peptides into large polypeptides is technically feasible, by ordered, stepwise condensation of peptides via the Fmoc procedure. But again, this approach is expensive and requires laborious, time consuming purification of products after each block condensation reaction.

The high expense of synthesizing the large numbers of polynucleotides and peptides needed to assemble genes and proteins is largely overcome by use of the segmented synthesis technology described in U.S. patent application Ser. No. 07/000,716, filed Jan 6, 1987), whereas the assembly of these biopolymer fragments into genes and proteins remains cumbersome and time consuming.

Another technology in genetic engineering and biotechnology is the use of enzymes to manipulate the genetic material in recombinant DNA research. Restriction endonucleases (enzymes that recognize and cleave DNA at specific sequences, 4-8 base pairs in length) are used to isolate specific regions of a chromosome, and DNA ligases (enzymes which join together fragments of DNA resulting from action of restriction enzymes) are used to "clone" the specific DNA fragments into extrachromosomal replicating genomes (plasmids or viral DNAs), known as "vectors" Berg, Science 213:296-303 (1981). The resulting

recombinant DNA is used to analyze the base sequence of a cloned fragment, or to produce large amounts of a protein coded for by a cloned gene. As discussed above, a powerful extension of this technology is the use of chemically synthesized duplex DNA fragments in place of a naturally occurring "restriction fragment" in formation of a recombinant DNA.

Although recombinant DNA technology represents a powerful tool in molecular biology research and genetic engineering, the labor intensive purification steps and/or analysis of numerous reaction products are required before a desired recombinant DNA product can be isolated.

Direct manipulation of proteins analogous to recombinant DNA methods (Offord, Protein Engineering, 1:151-157, (1987)), allows use of specific endopeptidases to excise specific segments from proteins, and then to replace these by synthetic pieces, chemically different from the natural peptides. This "recombinant protein technology" alos requires laborious purification steps and analysis of different reaction products in order to isolate the desired engineered protein.

Thus, despite the tremendous power of currently available genetic engineering techniques, further improvements are needed in the speed, efficiency and economy of biopolymer manipulations. Accordingly, due to the shortcomings of the present procedures, there exists a need for a process for rapid, low cost, efficient and accurate assembly of biopolymers from their subcomponents, and for rapid and convenient in vitro remodeling of biopolymer sequences, whereby isolation of the desired engineered biopolymer is achieved with a minimum of purification and analytical steps.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide an improved process for assembly of

biopolymers from subcomponenents thereof.

Another object of the present invention is to provide a process for more rapid assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides.

Yet another object of the present invention is to provide a more cost effective process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring less labor and materials.

A further object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring fewer purification and analytical steps.

Still another object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides, providing a greater yield of the desired end-product.

An additional object of the present invention is to provide a faster, more efficient and less costly process for assembly of peptides into polypeptides.

A further object of the present invention is to provide an improved process for replacement of a specific segment of a DNA molecule by an analogous, modified segment or by a different segment.

Yet another object of the present invention is to provide an improved process for replacement of a specific segment of a polypeptide molecule by an analogous, modified segment or by a new, unrelated segment.

Still another object of the present invention is to provide an improved process for deletion of one or more specific segments within a nucleic acid or protein.

An additional object of the present invention is to provide an improved process for insertion of one or more oligomeric segments at specific locations in a nucleic acid or protein.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention, an improved, general procedure for construction of biopolymers, comprising the following steps: (1) attachment of a biopolymer subcomponent to a solid phase support; (2) attachment of the next biopolymer sequence to one end of the support-bound component; (3) washing away of excess, unattached biopolymer sequences added in step (2); (4) ordered, stepwise attachment of oligomeric biopolymer sequences to the free end of the support-bound component (by repeated conduct of steps (2) and (3)), resulting in assembly of the biopolymer; and (5) release on the assembled biopolymer from the support. All steps in the foregoing process can be carried out in a suspension of the support, or alternatively, in a packed bed column fitted with porous means at both ends to provide a flow-through system. The biopolymer to be constructed by this process is chosen from among the group consisting of DNA (genes or gene segments), polypeptides (proteins), polysaccharides, or any other biopolymer composed of subsections that can be joined together. The "starting" biopolymer component initially attached to the support can range widely in length, for example 1-100 residues, the precise length being a matter of choice, but the support-bound starting component will typically be 10-50 residues in length. The nature of the solid phase support is a matter of choice, provided that the structure of the support does not sterically hinder the assembly of the desired high molecular weight biopolymer. The linkage of "starting" biopolymer component to the solid phase support is a matter of choice, readily achievable by one skilled in the art, using a variety of prior art methods. The nature of the stepwise linkage of oligomeric biopolymer segments during the assembly process, as well as the method of cleavage of final product from the support, will depend on the type of biopolymer being

constructed, details of which are given in the embodiments described below.

In accordance with one specific aspect of the present invention, there is provided an improved process for assembly of a gene (or gene fragment) from synthetic oligonucleotides, comprising the following steps: (1) attachment of a "starting" oligonucleotide to the solid phase support, at or near one of its two ends; (2) addition of a molar excess of the next oligonucleotide in the gene to be assembled, one end of the added oligonucleotide being complementary in base sequence to the free end of the support-bound oligomer, to form a molecule in which one end of the added oligonucleotide is base paired with the support-bound oligomer, leaving a single-stranded tail at the other end of the added oligomer; (3) washing away of the unannealed free oligonucleotides;, (4) repeated cycles of oligonucleotide addition/annealing/washing, carried out until the desired gene or gene fragment has been assembled; and (5) release of the assembled DNA from the support.

In step (1) of this preferred embodiment, the solid phase support is first derivatized with a nucleoside, then the "starting" oligonucleotide is synthesized on the solid phase support, using standard phosphate triester or phosphite triester procedures, the linkage of this synthesized oligonucleotide to the support being retained and utilized in the subsequent gene assembly. The solid phase support in this embodiment is preferably nonporous glass beads of small diameter (5-50 micrometers) or small diameter (5-50 micrometers) glass beads containing large diameter (1000-5000 A) pores. Derivatization of the glass beads with nucleoside can be achieved by a variety of prior art methods that are readily apparent to one skilled in the art. For example, the 3"-urethane linkage of a nucleoside to the glass via long chain alkylamine spacer arm (Sproat and Brown, Nucl.

Acids Res., 13:2979-2987, (1985)) can be employed to yield a solid phase support suitable for synthesis of the "starting" oligonucleotide by standard phosphoramidite or phosphate triester methods. The urethane linkage is largely retained during the deprotection of exocyclic amino groups, and can subsequently be utilized for solid phase gene assembly. Alternatively, solid phase synthesis of the "starting" oligonucleotide can proceed via the phosphoramidite method or phosphate triester method on glass beads derivatized with nucleoside via the standard 3"-O-succinyl linkage, provided that the sequence of the "starting" oligonucleotide is chosen to avoid nucleoside residues containing exocyclic amino groups, since the alkaline condition normally required for deprotection of exocyclic amino groups would cleave the DNA from the support. For employment of the O-succinyl linkage a starting oligonucleotide sequence consisting of thymidine and inosine residues would be appropriate.

In step (1) of another preferred embodiment (attachment of the "starting" oligonucleotide to the solid phase support) a preformed oligonucleotide is bonded to the support at or near one end. In this embodiment the nature of the solid phase support and the method of linkage between support and "starting" oligonucleotide are a matter of choice, readily acheivable by one skilled in the art, using procedures known in the art with the qualification that the structure of the solid phase must not sterically hinder the assembly of the gene and the linkage of the oligonucleotide to the support must withstand the conditions of stepwise annealing, used for subsequent gene assembly. The solid phase support for this embodiment of step (1) appropriately comprises nonporous latex microspheres derivatized by functional groups, such that chemical crosslinking or condensation can occur between the beads and a reactive group on one end of the starting oligonucleotide in the assembly.

Again, the linkage of oligonucleotide to the latex particles can be achieved by a variety of established procedures which would be apparent to one skilled in the art. For example, hydrazide-derivatized latex particles are readily linked to oligonucleotides derivatized at the 5'-end with aldehyde or carboxylic acid groups as described by Kremsky et al., Nucleic Acids Res., 15:2891-2909, (1 987)). Alternatively, alkylamine-derivatized beads may be linked to alkylamine-derivatized oligonucleotides, using a bifunctional crosslinking reagent such as disuccinimedyl suberate as described by Pilch & Czech, J. Biol. Chem. 254:3375-3381 (1979). In addition, alkylamine-derivatized latex particles can first be linked to avidin or streptavidin by glutaraldehyde activation such as described by Goodfried et al., Science 144:1344 (1964), then the first oligonucleotide in the assembly, labeled with biotin at its 5'-end, will attach to the beads through the well known tight avidin-biotin affinity.

The determination of whether the 5' or 3' end of the starting oligonucleotide (5' or 3') is attached to the solid phase support (which dictates the directionality of the gene assembly) is entirely a matter of choice, except when the linkage of starting oligonucleotide to the support is more conveniently achieved at one particular end of the oligonucleotide.

Step (2) in the gene assembly process (annealing of the next oligonucleotide in the desired gene or gene fragment) may be carried out under any of the standard annealing conditions known to those skilled in the art, for example, incubation at 50-65° C in the presence of 0.2-1 M NaCl or KCI, or incubation at 37° C in the presence of 0.2-1 M salt plus 50% formamide. The base sequence of the oligonucleotides may be chosen to satisfy the following specifications: (1) The desired base sequence of gene is generated by the assembly process; (2)

The extent of complementary overlap (yielding a duplex segment holding the added oligonucleotide to the support-bound component) may be any length so long as to provide the required stability of the association is provided. In a preferred embodiment the complementary overlap sequence will be at least about 10 bases and may be up to 50 bases; (iii) The length of protruding single-stranded "tail" after annealing is preferably at least 10 bases (the length yielding stable base pairing with the subsequently added oligonucleotide); (iv) Oligonucleotide sequences are preferably chosen to avoid secondary structure within the oligonucleotides (intrastrand base pairing resulting in hairpins), which may interfere with annealing of the added oligonucleotide to the support-bound component; and (v) The sequences are chosen to avoid the production of more than one annealing product (through a multiplicity of base pairing possibilities).

Step (3) in the gene assembly (washing away of excess, unannealed added oligonucleotide) can conveniently be achieved by flow of solvent past the solid phase support, for example through a reaction chamber containing porous members at both ends. Alternatively, step (3) can be accomplished by a series of brief centrifugation/ decanting steps in microcentrifuge tubes.

In step (4) (ordered, stepwise repeat of steps (2) and (3) to build up the desired gene or gene fragment) of one embodiment of the present invention, the oligonucleotides assembled are designed to yield a completely duplex DNA with strand interruptions at positions alternating along both strands. In another embodiment, the oligonucleotides are designed for assembly of a partially duplex DNA molecule, in which single-stranded gaps exist in alternating positions along both strands. These gaps may be filled in by action of a DNA polymerase <u>in vitro</u>.

Step (4) may also be carried out by addition of several (eg., about 2-5) oligonucleotides in each annealing step. Although this procedure potentially reduces the total number of annealing steps required for assembly of the desired gene or gene fragment, care must be taken to insure that multiple products of annealing are not generated, i.e., that all support-bound assemblies generate the identical, desired duplex DNA sequence.

Step (5) (release of the assembled gene from the support) is carried out by means chosen to be compatible with the nature of the linkage of DNA to the support and the structure of the assembled DNA. In one preferred embodiment the stepwise annealing is carried out with all oligonucleotides being 5'-phosphorylated except for one, such that a completely duplex DNA is formed in which all strand interruptions can be sealed by use of DNA ligase, except for a single nonligatable strand interruption adjacent to the support-linked oligonucleotide. Then the contiguous duplex segment may then be removed from the support by brief heating to 80-100° C. Alternatively, the nonligatable strand interruption adjacent to the support can be made by leaving a gap of one or more nucleoside residues at this position in the assembled DNA.

In step 5 of another preferred embodiment, appropriate oligonucleotides are selected for the assembly such that a duplex DNA segment containing a restriction enzyme recognition sequence is generated between the gene or gene fragment and the support, such that release of the DNA from the support can be conveniently achieved via cleavage by the restriction endonuclease.

In all embodiments of the gene assembly process, the DNA released from the support is conveniently cloned into a vector for expression in cells and DNA sequence analysis.

In accordance with another aspect of the present invention, there is provided a process for assembly of

polypeptides, comprising the following steps: (1) attachment of a peptide to a solid phase support material; (2) stepwise end-to-end block condensation or ligation of peptides to the initial support-bound peptide, alternating with washing steps, to construct a longer polypeptide, and (3) cleavage of the polypeptide from the support.

In a particularly preferred embodiment, the solid phase support comprises small diameter (5-50 micrometers) nonporous glass beads to which the first amino acid residue is covalently attached via a long chain alkylamine spacer arm. In another preferred embodiment, the solid phase support comprises small diameter (5-50 micrometers) glass beads containing pores of large diameter (1000-5000 A). Both supports serve to avoid steric hindrance during the assembly of long polypeptides. The peptide can be attached to the supports after the synthesis of the peptide, or alternatively, the glass beads can be first derivatized with an amino acid residue, then used for solid phase peptide synthesis to create a support-bound peptide which is subsequently elongated in the assembly process. Although the preceding embodiments give examples of the kind of solid phase support and the type of linkage of peptide to the support which may be utilized, these parameters are a matter of choice. One skilled in the art could devise alternate peptide-linked supports that possess the favorable steric properties suitable for polypeptide assembly. Peptide-linked nonporous latex microspheres may also be used as a solid phase support.

In step (2) of one preferred embodiment of the solid phase polypeptide assembly (stepwise block condensation) of the present invention, the stepwise condensation of amino terminus-protected peptides onto the free amino terminus of a peptide linked to the support via its carboxy terminus, is carried out using the standard Fmoc procedure. In another embodiment, stepwise block condensation on the solid phase support is performed

chemically, by use of a peptide bond-forming reagent such as dichlorophenol, or enzymatically, by "reverse proteolysis" (Offord, Protein Engineering, 1:151-157, (1987)).

In accordance with still another aspect of the present invention, there is provide a general procedure for remodeling of biopolymer sequences on a solid phase support, comprising the following steps: (1) attachment of a high molecular weight biopolymer at one or more positions in the biopolymer sequence to a solid phase support; (2) excision of a specific segment of the biopolymer; (3) washing away of the cleaving agents and excised biopolymer segment; (4) addition of a chemically synthesized biopolymer sequence or a fragment isolated from natural sources and specific insertion of the added segment into the biopolymer sequence to replace the excised segment; (5) washing away of excess added biopolymer segment and bond-reforming agents;, and (6) cleavage of remodeled biopolymer from the support. The foregoing general procedure for biopolymer remodeling can also be used to insert or delete biopolymer segments at specific positions in the biopolymer sequence.

In step (1) of biopolymer remodeling the nature of the solid phase support and means for its attachment to the support are a matter of choice, depending on the structure of biopolymer, and would be readily chosen from existing applications by one skilled in the art. For example, avidin-coated beads could be used to tightly bind biotin-labeled DNA or biotin-labeled protein. Alternatively, a specific antibody-bound support could be used to bind an epitope in a protein or nucleic acid. Also, a support-linked oligonucleotide (preferably 20-50 residues in length) could be used to link a single-stranded DNA molecule to the support, via hydrogen bonded base pairing. In addition, a reversible crosslinking agent could be used to connect chemically reactive groups in the biopolymer and support.

Site-specific cleavage of the biopolymer (step (2) of solid phase biopolymer remodeling) is preferably achieved by enzymatic means, utilizing one or more restriction endonucleases in the case of DNA, or specific endopeptidases in the case of protein. In the specific case of single-stranded DNA attached to the support, cleavage by restriction endonuclease can be achieved by adding oligonucleotides which anneal to the DNA to provide short duplex regions containing the enzyme"s recognition sequence. Also, a specific chemical cleavage means (for example, cleavage of protein by cyanogen bromide) can also be employed in step (2).

In step (3) of solid phase biopolymer remodeling, the cleaving agents and excised biopolymer segment are washed from the support, preferably by flow of solvent past the support-bound biopolymer contained within a chamber fitted with porous means at both ends. Alternatively, repeated brief centrifugation/decantation steps can be used in step (3) for support-bound biopolymer contained within microcentrifuge tubes.

In step (4) of solid phase biopolymer remodeling, a "replacement" biopolymer segment is added, preferably in molar excess over support-bound biopolymer, along with an appropriate bond-reforming agent, to achieve replacement of the biopolymer segment excised in step (2) by the segment added in step (4). For example, in remodeling of DNA, a restriction fragment isolated from natural sources or a chemically synthesized duplex segment containing the appropriate termini may be added, and ligated into the position previously occupied by the segment excised in step (2), by the action of DNA ligase. In the case of protein, replacement of the excised segment by an added peptide can be achieved enzymatically, by "reverse proteolysis" catalyzed by specific endopeptidases under reaction conditions such as disclosed in Offord, Protein Engineering, 1:151-157, (1987)), or can be achieved

chemically, by action of a peptide bond-forming agent such as dichlorophenol.

Washing away of excess reaction components from the support-bound biopolymer (step (5)) may be achieved by the same means is in step (3).

Cleavage of the remodeled biopolymer from the support (step (6)) can be carried out by a variety of means that would be apparent to one skilled in the art, the method of choice depending on the nature of the solid phase support and biopolymer and the type of linkage between them. For example, for biopolymers attached to the support via the avidin:SS-biotin affinity, the linkage is readily broken by addition of buffer containing 100 mM dithiothreitol Shimkus et al., Proc. Natl. Acad. Sci. (USA) 82:2593-2597 (1985), dissociation of remodeled biopolymer from an antibody affinity support can be achieved by common protein denaturants, and release of a DNA molecule base paired to a support-bound oligonucleotide can be achieved by brief heating to 80-100° C.

Further objects, features and advantages of the present invention will become apparent from a review of the detailed description of the preferred embodiments which follows, in view of the drawings, a brief description of which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the solid phase biopolymer assembly process as applied to construction of a gene or gene fragment.

Figure 2 is a schematic diagram of the solid phase biopolymer assembly process as applied to construction of a polypeptide.

Figure 3 is a schematic diagram of the general procedure for remodeling of a biopolymer on a solid phase support.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention will be described in detail by reference to the drawings. Although the embodiments described herein refer to a few specific uses of the present invention, many variations of the process are also within the scope of the present invention which will be obvious to one skilled in the art.

Figure 1 illustrates the concept of assembly of a gene or gene fragment on a solid phase support. A "starting"' oligonucleotide 3 is first attached to a solid phase support 1. As discussed previously, the precise nature of the support 1 and the type of linkage 2 between the starting oligonucleotide and the support are a matter of choice, and are readily known to those of skill in the art. It is essential, however, that the geometry of the solid phase support is such that assembly of the gene is not sterically hindered, as it would be with most currently used solid phase support materials. To satisfy this requirement, a solid phase support consisting of small diameter (5-50 micrometers) nonporous glass beads, or alternatively, macroporous beads (5-50 micrometers in diameter) with very large pores (1000-5000 A) are recommended for use in solid phase assembly of genes.

The linkage 2 of the starting oligonucleotide to the beads can take a variety of forms, readily known to those of skill in the art. The following examples of suitable linkages are given for illustrative purposes, and it is emphasized that alternative linkages, readily apparent to one skilled in the art, are also within the scope of the present invention.

One suitable linkage 2 to glass beads is the urethane linkage described by Sproat and Brown supra and incorporated herein by reference. The urethane linkage is ideally suited for a synthesis of a starting oligonucleotide of any base sequence prior to gene

assembly, since the urethane linkage is more stable than the acyl linkages protecting the exocyclic amino groups of A, G and C, such that the oligonucleotide will be retained on the support under the conditions used for deblocking the bases.

If the starting oligonucleotide in gene assembly is designed to contain a sequence of I (inosine) and T (thymidine) nucleosides, then the standard 3"-0-succinyl linkage can be used to synthesize the starting oligonucleotide, because an alkaline base-deblocking step (which would hydrolyze the 3'-0-succinyl linkage) would not be required after synthesis of oligo(I,T).

Several procedures are available for linkage of a presynthesized starting oligonucleotide to the surface of solid latex microspheres, providing a support-bound oligonucleotide suitable for gene assembly. For example, the well-known tight avidin-biotin affinity may be employed, by covalently linking avidin to small alkylamine-derivatized latex beads (0.1-10 microns in diameter) by the glutaraldehyde activation or other methods known in the art, producing avidin-coated beads that will bind a 5'-biotin-labeled oligonucleotide.

Latex microspheres may also be covalently attached to the starting oligonucleotide for gene assembly by other methods, including the use of a homobifunctional crosslinking agent such as disuccinimedyl suberate to link alkylamine-derivatized latex beads with 5"-alkylamine-derivatized oligonucleotide, linkage of hydrazide-derivatized latex beads to a 5"-aldehyde-oligonucleotide or to a 5'-carboxylate-oligonucleotide and other such linkage methods are known in the art (Kremsky et al., Sproat and Brown, Shimkus et al., Pilch and Czech, and Goodfriend et al., supra, all incorporated herein by reference.

The protocol for solid phase gene assembly illustrated in Fig. 1 calls for performance of a series of

stepwise annealings, using oligonucleotides 4, 5, 6, 7, and n to build up the desired gene or gene fragment. The degree of base "'overlap" at each annealing step will preferably result in formation of at least twenty base pairs between added oligonucleotide and support-bound single-stranded "tail." In the example shown in Fig. 1, the starting oligonucleotide is attached to the support via its 3"-end, and is non-phosphorylated. Oligonucleotides added in the stepwise annealing reactions are 5"-phosphorylated and designed to form a fully double-stranded assembled DNA (containing no single-stranded "gaps"), in which the strand interruptions (5'-phosphate adjacent to 3'-OH) in one strand are located at approximately the midpoint of the oligonucleotides comprising the other strand. Under these conditions, the '"nicks'" can be enzymatically sealed by action of DNA ligase, prior to release of the assembled gene from the support.

The stepwise annealing in gene assembly is preferably carried out in a small volume (eg., 0.02-0.10 ml), with the solid phase support kept in suspension by gentle agitation (except with submicron latex particles, which are kept in suspension by Brownian motion). The quantity of starting oligonucleotide attached to the support can vary widely, for example, 0.01-1.0 micromoles per gram of beads. At such a '"loading capacity'" of the beads, essentially quantitative stepwise annealing would occur within a few minutes under the following reaction conditions (0.10 ml annealing volume): 50 mM potassium or sodium phosphate, pH 7.5, 400 mM KCl or NaCl, 0. 1 -1.0 nanomole of support-bound oligonucleotide, 0.2-2.0 nanomole of added oligonucleotide, 50-60° C. Alternatively, an identical reaction mixture, containing addition of 50% formamide, could be incubated at 37° C. Under the foregoing conditions the concentration of annealing DNA (assuming 20 base pair overlap) is 20-200

micrograms per ml. The quantity of each added oligonucleotide in the gene assembly is very low, accommodating the use of inexpensive methods of oligonucleotide synthesis that provide low yields of purified product.

The stepwise annealing of one oligonucleotide at a time is recommended, to insure that annealing occurs specifically and quantitatively. However, it is possible that the procedure illustrated in Fig. 1 could be successfully adapted to the addition of several oligonucleotides at a time, thereby requiring fewer steps to assemble a gene. However, even with individual annealings, a 1000 base pair gene could be assembled within six hours, assuming assembly of fifty 40mers, five minute annealing time and two minute washing time.

The washing step carried out after each annealing reaction, which removes excess unannealed oligonucleotides, thus assuring formation of the desired annealing product in each cycle, is preferably carried out by flow of solvent (eg., annealing buffer) past the support, which may be provided for by housing the solid phase support within a reaction chamber having porous means at both ends such as that disclosed in U.S. Patent application Serial No. 000,716.

Alternatively, 2-3 brief centrifugation/ decantation steps may be carried out (with support held within a microcentrifuge tube) to achieve satisfactory washing.

Obviously, in order to obtain the correct assembly and structure of a gene it is critical that the oligonucleotides added at each step be homogeneous.

After the completion of the gene assembly the DNA product must be released from the support. In the example shown in Fig. 1, this is simply achieved by a brief heating step (80-90° C), which denatures the short duplex section holding the assembled gene to the support, without

causing complete denaturation of the long assembled duplex DNA (the latter having been converted to contiguous long strands by action of DNA ligase). The unsealed strand interruption at the beginning of the assembled gene (at the junction of oligonucleotides 3 and 5 in Fig. 1, resulting from the absence of a 5'-phosphate on the support-bound starting oligonucleotide) could also be arranged by formation of a nonligatable "gap" of at least one base at this position.

Alternatively, the assembled gene or gene fragment could conveniently be released from the support by action of a restriction endonuclease, provided that its recognition sequence were designed into the duplex DNA near the support (eg., within the duplex segment formed by oligonucleotide 4 in Fig. 1). In the latter case, 5'-phosphorylation of the oligonucleotides used to assemble the gene may be optional.

As illustrated in Fig. 1, the released gene (or gene fragment) 9 may be used for any purpose, i.e., it may be subsequently cloned into a vector for sequence analysis, expression (production of protein encoded by the gene), etc.

To achieve maximal benefit from the gene synthesis and/or assembly of the present invention, the base sequence of the oligonucleotides to be assembled should be carefully planned, with the following considerations in mind: (1) The sequences should be designed to avoid formation of hairpins of four or more base pairs within the oligonucleotides, which may interfere with efficient intermolecular base pairing during the annealing steps. (2) Sequences that are commonly associated with poor coupling efficiency during the chemical synthesis (such as four or more consecutive G residues) should be avoided. (3) Sequences that introduce "rare codons" into a gene should be avoided, if possible, if the aim is to achieve high levels of gene expression.

"Rare codons" are those nucleotide sequences which are rarely found in nature and thus may not be properly translated in some hosts. (4) Oligonucleotides may be designed to generate unique restriction sites within the assembled gene to facilitate subsequent manipulations by recombinant DNA techniques. For example, if mutations are found at intervals within a chemically synthesized gene, the existence of unique restriction sites permits cleavage of individual cloned genes with restriction endonucleases, and recombination to form the desired mutant-free gene.

The length of a duplex DNA that may be assembled in the manner illustrated in Fig. 1 ranges widely, from less than a hundred base pairs up to thousands of base pairs. Because labor-intensive purification and analysis of intermediates in the gene assembly are avoided by use of the solid phase approach, time and expense associated with gene construction are greatly reduced by use of the present invention. Furthermore, the high efficiency of the process permits the use of very small quantities of DNA, further reducing the cost of gene synthesis. An average size gene may be synthesized, assembled and cloned into an expression vector within a period of one week, at a total cost for materials and labor of less than $1,000 if the segmented DNA synthesis device disclosed and claimed in U.S. Patent Application No. 000,716, filed Jan. 6, 1987 is used to synthesize the oligonucleotides at 50 nanomole scale, and then the present invention were used to assemble the gene. The cost of preparing the same gene by conventional methods would be $20,000 to $50,000 and would typically require about two months work.

The present invention although exemplified as a means for gene assembly, is equally applicable to construction of other biopolymers, including polypeptides and polysaccharides. The method of the present invention used for polypeptide assembly is shown in Figure 2. A protein molecule could be constructed by ordered, stepwise

chemical condensation between the free amino terminus of a peptide linked to the support via its carboxy terminus and successively added amino terminus-protected peptides, using standard Fmoc chemical condensation known to those of skill in the art.

The present invention may also be applied to remodeling of a biopolymer, a multistep process which, if carried out in solution by conventional means, frequently requires time-consuming and labor-intensive purification and analytical steps before the desired end product is obtained. By performing the same manipulations on a solid phase support rather than in solution, the need for purification and analysis of intermediate products is avoided, thus saving time and labor. The advantage of carrying out a biopolymer reconstruction on a solid phase support is illustrated in Figure 3.

Biopolymer 11 is first attached to a solid phase support 1 at one or more positions in the biopolymer sequence. As explained previously for the process of biopolymer assembly, the precise nature of the solid phase support and the method of linkage of biopolymer thereto are entirely a matter of choice, the only constraint being that the structure of the solid phase support must not restrict accessibility of reaction components to the biopolymer. Suitable solid phase supports, types of linkages, means for washing away reaction components and means for ultimate release of biopolymers from the supports such as those described previously for biopolymer assembly may be utilized.

The support-bound biopolymer 11 (for example, a double-stranded plasmid DNA) is treated with at least one agent (for example, restriction endonuclease(s)) to produce cleavage at one or more specific sites 13 within the biopolymer sequence. If the biopolymer is cleaved at two specific sites, one or more specific fragment(s) 12 were released (for example, a restriction fragment). The

released fragment(s) 12 and cleaving agent(s) are conveniently washed away, as described previously for biopolymer assembly, then a replacement fragment 14 (for example, a restriction fragment or synthetic duplex DNA) is added, and the bonds are reformed (for example, by DNA ligase), producing the remodeled biopolymer 15.

This procedure may be used to produce a deletion within the biopolymer, by reforming the bonds after removal of the released segment 12, without adding back a replacement segment.

In addition, this procedure may be used to insert an additional biopolymer segment into a specific location within the support-bound biopolymer, by cleaving at a single site within the biopolymer, and then attaching a biopolymer segment at this position (for example, insertion of a "'foreign'" duplex segment or synthetic duplex DNA at a unique restriction site within a cloning vector, to produce a recombinant DNA).

The application of the present invention to recombinant DNA technology is advantageous, because of the elimination of time-consuming purification steps that are typically carried out in order to remove a released DNA segment before replacing it with another sequence.

EXAMPLE 1

Remodeling of Bacteriophage M13 Vector

A specific application of the present invention for manipulation of single-stranded circular DNA (such as a bacteriophage M13 vector) is now given, with reference to Fig. 3. A circular, single-stranded phage DNA 11 is conveniently attached to the solid phase support via base pairing with a support-bound synthetic oligonucleotide (20-50 bases in length, complementary to a specific region within the single-stranded vector). Next, two synthetic oligonucleotides (eg., 20mers) are added and allowed to anneal with the vector sequence, producing short duplex

regions containing the restriction sites 13.

Restriction endonuclease(s) are then used to cleave out a segment 12 of the DNA between the restriction sites, and the restriction enzyme(s) and released fragment are washed away. Then a replacement fragment 14 (containing short duplex regions at the ends, with identical termini as in the fragment removed) is added and joined to the support-bound DNA by action of DNA ligase. When the cleavage of support-bound single-stranded vector is carried out at a single, unique position, a restriction fragment or synthetic DNA will be inserted at this position. After these manipulations are performed, the DNA can be made completely double-stranded by action of a DNA polymerase, and as polymerization proceeds through the short duplex region connecting the vector to the support-bound oligonucleotide, the vector is released from the former, by the well-known "'strand displacement" phenomenon. Finally, the DNA may be converted to closed circular form by action of DNA ligase.

EXAMPLE 2

Synthesis and assembly of a segment of the E. coli lacl gene.

Solid Phase Support:

The support used for both synthesis of the "starting" oligonucleotide and for subsequent gene assembly consisted of long chain alkylamine-derivatized solid glass beads of 6 micrometer diameter, derivatized with 5'-trityl,2'-deoxythymidine to form the 3'-O-urethane linkage (Sproat and Brown, 1985). The "loading" capacity of the support, determined by HPLC analysis following release of nucleoside from support by 24 hour treatment at 55° C in concentrated ammonium hydroxide, was 2.2 micromoles nucleoside per gram of support.

Synthesis of Oligonucleotides:

45 mg of support was derivatized by the method described by Sproat and Brown with 0.1 umoles of a first nucleoside (A). A non-phosphorylated oligonucleotide (3'-AAAAAAAAAAAAAAAAAAGCGTCGCACGCT-5') was synthesized on the support by the phosphoramidite method (Beaucage & Caruthers, 1981), using a Milligen 7500 DNA synthesizer. Following the final detritylation step, the support material was placed into a glass vial and treated with 1.5 ml concentrated ammonium hydroxide at 55° C. for 1 hour to remove the protecting groups from the exocyclic amino groups. The support material was then placed into a 1.5 ml Eppendorf tube and washed five times (by centrifugation/decantation) with annealing buffer (as specified below).

Annealing Conditions for Gene Assembly:

To a 1.5 ml Eppendorf tube was added 0.5 mg oligonucleotide-support (approx. 1 nmole), 2 nanomole of the 5'-phosphorylated oligonucleotide,

5'-pTTTCGCAGCGTGCGAGCGTGCCCGGGTGGT-3'

and sufficient annealing buffer (50 mM kH_2PO_4,pH 7.5, 400 mM KCl, 1mM EDTA) to bring the volume to 0.10 m. The tube was incubated at 55° C. for 5 minutes, with occasional gentle agitation, then the tube was centriguged for 1 minute in an Eppendorf microcentrifuge and the beads were washed twice with 1 ml annealing buffer.

After the first annealing and washing steps the product is:

Sup-U-O-3'-AAAAAAAAAAAAAAAAAAGCGTCGCACGCT-5'
5'-pTTTCGCAGCGTGCGAGCGTGCCCGGGTGGT

The annealing/washing cycle was repeated, using 2 nanomole each of the following 5'-phosphorylated oligonucleotides in succession:

3'-CGCACGGGCCCACCACTTGGTCCGGTCGGTp-5' (3)

5'-pGAACCAGGCCAGCCACGTTTCTGCGAAAAC-3' (4)

3'-GCAAAGACGCTTTTGAGCTp-5'(5)

The final product, the lacl gene fragment coupled to the solid support is depicted in Figure 4.

Ligation and Cleavage of Duplex DNA From the Support:

The support was washed twice with ligase buffer (50 mM Tris1-HCl, pH 7.8, 20mM dithiothreitol, 10mM $MgCl_2$, 1mM ATP, 0.05 mg/ml bovine serum albumin), then resuspended in 0.098 ml ligase buffer. Two microliters of DNA ligase was added (New England Biolabs, high specific activity grade). After incubation for 30 minutes at 37° C., with occasional gentle agitation, the support was then washed twice with Ava 1 buffer (10mM Tris-HCl, pH 8, 50mM NaCl, 10mM $MgCl_2$, 5mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin), then resuspended in 0.098 ml of this buffer. Two microliters of restriction endonuclease Ava 1 (New England Biolabs) were added, and the mixture was incubated at 37° C. for 30 minutes to cleave the DNA from the support. The tube was centriguged and the supernatant was collected. The beads were washed twice with 0.1 ml Ava 1 buffer, and the DNA in the combined supernatants were ethanol precipitated and dissolved in 0.1 ml of 10mM Tris-HCl, pH 7.5 containing 1mM EDTA.

Cloning and Analysis of the Synthetic Gene Segment:

Approx. 1 nmol of the duplex lacl gene fragment prepared above was mixed with 1 nmol of M13-lacl-SAXB which had been previously cleaved with Ava 1 and passed over a Sepharose 2B column to remove the 40-bp segment of the lacl gene. The DNA was ligated with 4% DNA ligase as described above. The DNA was then transfected into E. coli strain JM107 and progeny phage were plated into E.

coli strain PD8. This genetic system provides the opportunity to assess the possible generation of mutations during the chemical synthesis of the lacl gene fragment (mutations are seen as blue plaques in the absence of inducer). The frequency of mutations in this experiment was undetectable over the spontaneous frequency.

The DNA of the semi-synthetic M13-lacl was sequenced by the "dideoxy" method and found to contain the desired wild-type sequence in the region of the chemical synthesis. Thus, the DNA duplex synthesized by the method of the present invention was identical in both sequence and mutation frequency to that of the naturally occurring wild-type lacl sequence.

Although Fig. 2 illustrates a general procedure for biopolymer reconstruction (remodeling), many variations of the process, specific for different bipolymers and different types of manipulations thereupon, will be evident to one skilled in the art and are within the scope of the present invention. For example, the solid phase remodeling process could be used to replace a specific segment of a protein with a different, modified segment, a solid phase "recombinant protein" technique analogous to the solid phase recombinant DNA example discussed previously.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The components, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope of the present invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A process for assembling a biopolymer from oligomeric subcomponents thereof, comprising the steps of:

(1) attaching one end of a biopolymer oligomeric subcomponent to a solid phase support;

(2) attaching a next oligomeric sequence at or near the free end of the support-bound component;

(3) removing excess, unattached oligomeric sequences;

(4) repeating steps 2 and 3 for the ordered, stepwise attachment of oligomeric biopolymer subcomponents at or near the free end of the support-bound component resulting in assembly of the biopolymer; and

(5) releasing the assembled biopolymer from said support.

2. A process for assembling a gene from synthetic oligonucleotides comprising the steps of:

(1) attaching a first oligonucleotide to a solid phase support at or near a first end of said oligonucleotide;

(2) removing excess, unattached oligonucleotide;

(3) hybridizing a next oligonucleotide containing at least 5-100 base pairs complementary to the free end of said bound oligonucleotide wherein said next oligonucleotide contains at least 5-100 base pairs more than said complementary sequence;

(4) removing excess, unattached oligonucleotide;

(5) repeating steps (3) and (4), for the ordered, stepwise hybridization of oligonucleotide sequences at or near the free end of the support-bound component; and

(6) releasing the assembled gene or gene fragment from the support.

3. A process as claimed in claim 1, wherein said biopolymer is selected from the group consisting of gene or portion thereof, a genome or portion thereof, a ribonucleic acid, a polypeptide, and a polysaccharide.

4. A process as claimed in claim 1, wherein the successively added components become noncovalently attached to the support-bound biopolymer component by noncovalent forces, including hydrogen bonding, electrostatic interaction or hydrophobic interaction.

5. A process as claimed in claim 1, wherein the successively added components become covalently bonded to the support-bound biopolymer by means of chemical or enzymatic reaction.

6. A process as claimed in claim 1, wherein said solid phase support consists of a nonporous, particulate material selected from the group consisting of silica (glass), latex, polystyrene and plastic.

7. A process as claimed in claim 1, wherein said solid phase support consists of a macroporous material containing intramatrix spaces (pores) large enough to prevent steric hindrance of biopolymeric assembly.

8. A process for remodeling of a biopolymer partial substitution or modification, comprising:

a solid phase support material;

means for attachment of a biopolymer to said support material at one or more positions in the biopolymer sequence;

means for excision of at least one specific segment of the support-bound biopolymer, the excised segment being washed away, then replaced by addition of a modified, replacement segment which becomes specifically attached to the support-bound biopolymer, thus providing for segmental substitution of the biopolymer; and

means for removal of the remodeled biopolymer from the support.

9. A process as claimed in claim 8, wherein the support-bound biopolymer is a double-stranded DNA molecule, the means for excision comprises a restriction endonuclease, the replacement segment comprising a double-stranded DNA segment, produced synthetically or isolated from natural sources, and the means for rejoining of the replacement segment to the support-bound DNA comprising DNA ligase.

10. A process as claimed in claim 8, wherein the support-bound biopolymer is a single-stranded DNA molecule, the means for excision comprises addition of one or more restriction endonucleases and one or more synthetic oligonucleotides which anneal to the single-stranded DNA to create cleavage sites for action of the restriction endonuclease(s), the replacement segment comprising a double-stranded DNA segment, produced synthetically or isolated from natural sources, and the means for rejoining of the replacement segment to the support-bound DNA comprising DNA ligase.

11. A process as claimed in claim 8, wherein the support-bound biopolymer is a polypeptide and the means for excision comprises a specific endopeptidase, the replacement segment comprises a peptide, produced synthetically or isolated from natural sources, and

wherein the means for linkage of the added segment to the support-bound polypeptide comprises chemical condensation or enzymatic ligation.

12. A process as claimed in claim 8, wherein rejoining of the biopolymer is carried out without addition of a replacement segment, resulting in deletion of one or more segments from the biopolymer.

13. A process as claimed in claim 8, wherein cleavage of the biopolymer occurs at a single site, and insertion of added biopolymer segment occurs at this site.

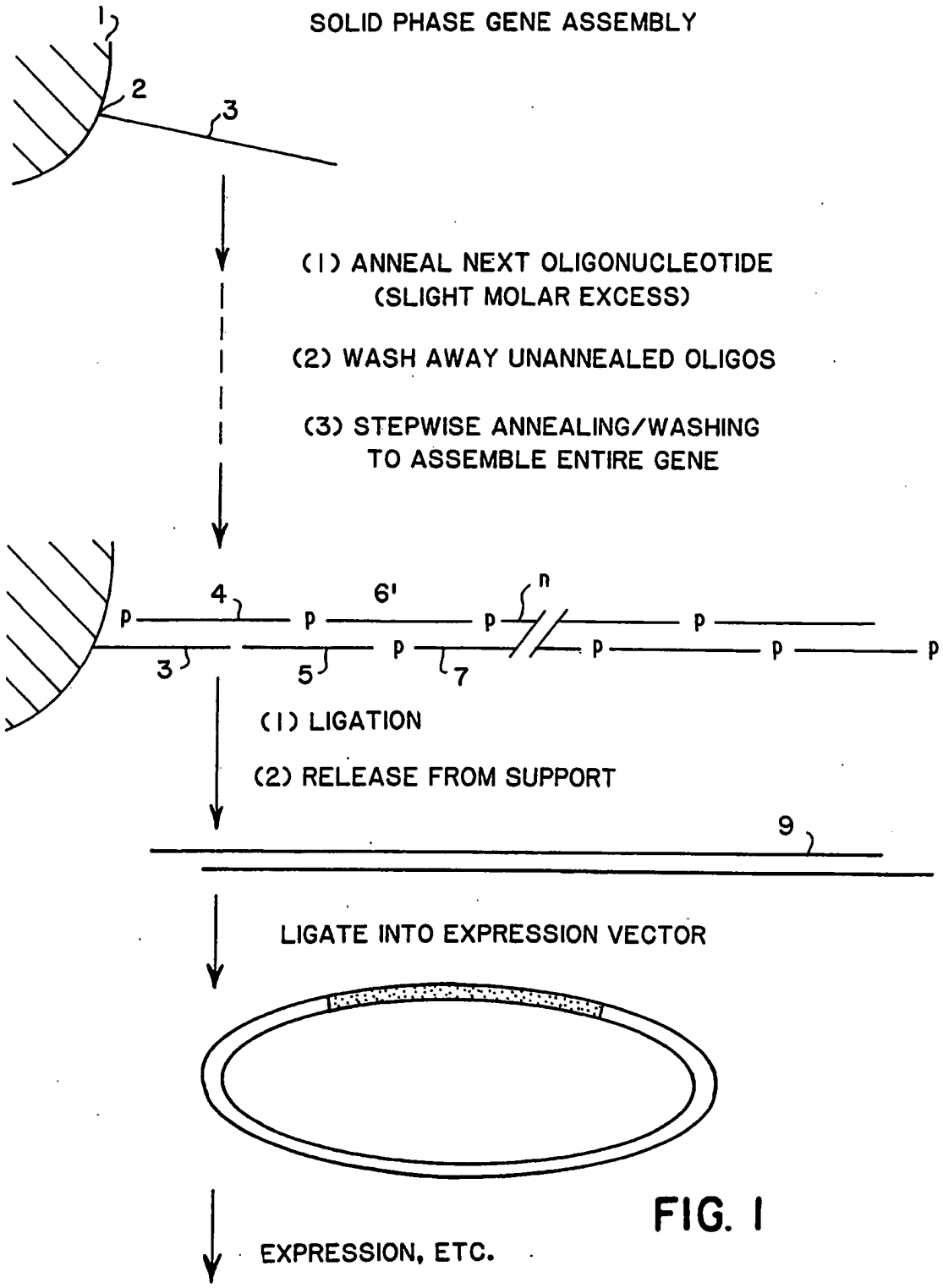
SOLID PHASE GENE ASSEMBLY
1
2
3
(1) ANNEAL NEXT OLIGONUCLEOTIDE
(SLIGHT MOLAR EXCESS)
(2) WASH AWAY UNANNEALED OLIGOS
(3) STEPWISE ANNEALING/WASHING
TO ASSEMBLE ENTIRE GENE
4
6'
n
p
3
5
7
(1) LIGATION
(2) RELEASE FROM SUPPORT
9
LIGATE INTO EXPRESSION VECTOR
EXPRESSION, ETC.

FIG. 1

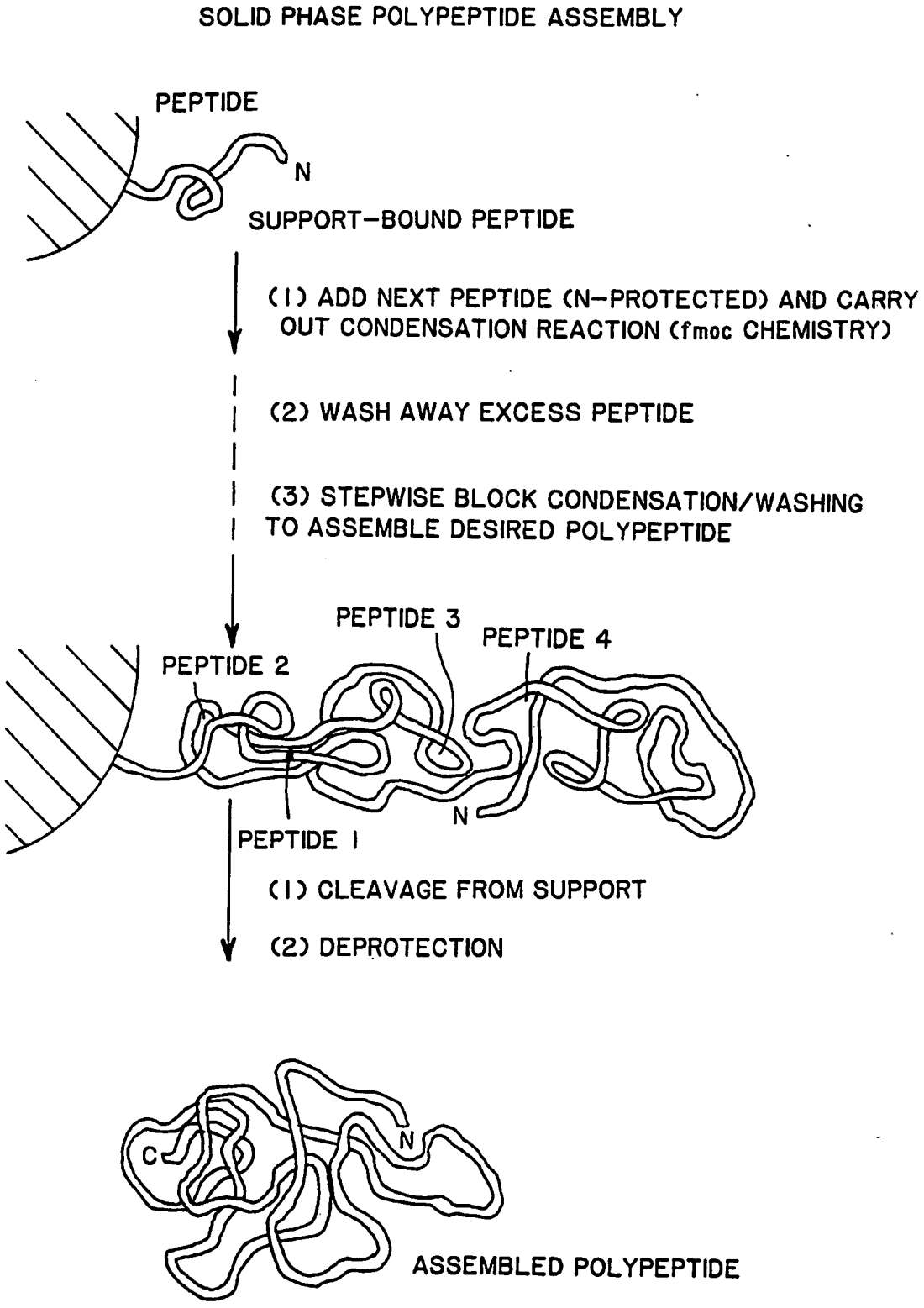
SOLID PHASE POLYPEPTIDE ASSEMBLY
PEPTIDE
N
SUPPORT-BOUND PEPTIDE
(1) ADD NEXT PEPTIDE (N-PROTECTED) AND CARRY OUT CONDENSATION REACTION (fmoc CHEMISTRY)
(2) WASH AWAY EXCESS PEPTIDE
(3) STEPWISE BLOCK CONDENSATION/WASHING TO ASSEMBLE DESIRED POLYPEPTIDE
PEPTIDE 3
PEPTIDE 4
PEPTIDE 2
N
PEPTIDE 1
(1) CLEAVAGE FROM SUPPORT
(2) DEPROTECTION
C
N
ASSEMBLED POLYPEPTIDE

FIG. 2

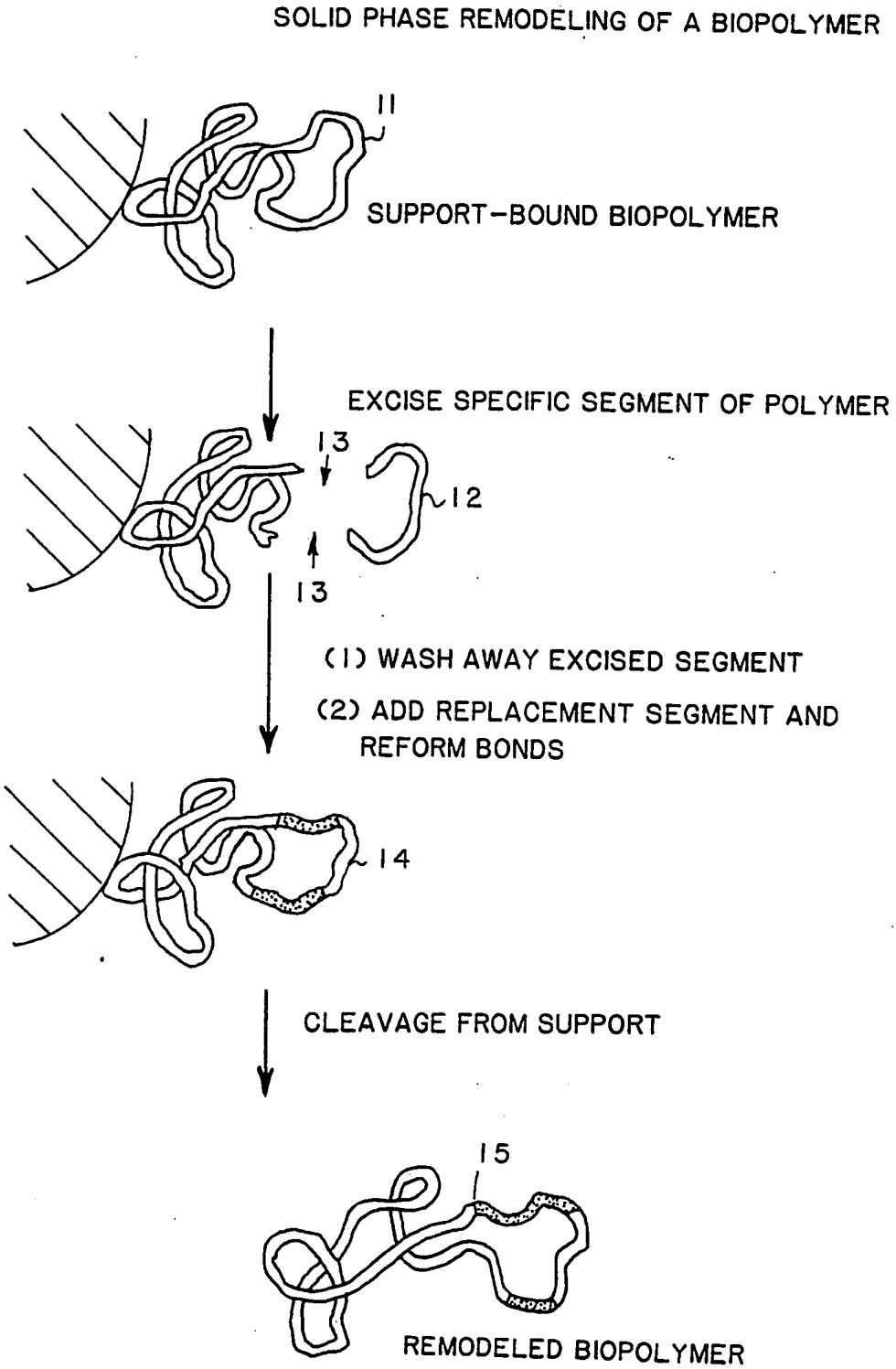
SOLID PHASE REMODELING OF A BIOPOLYMER
11
SUPPORT-BOUND BIOPOLYMER
EXCISE SPECIFIC SEGMENT OF POLYMER
13
12
13
(1) WASH AWAY EXCISED SEGMENT
(2) ADD REPLACEMENT SEGMENT AND REFORM BONDS
14
CLEAVAGE FROM SUPPORT
15
REMODELED BIOPOLYMER

FIG. 3

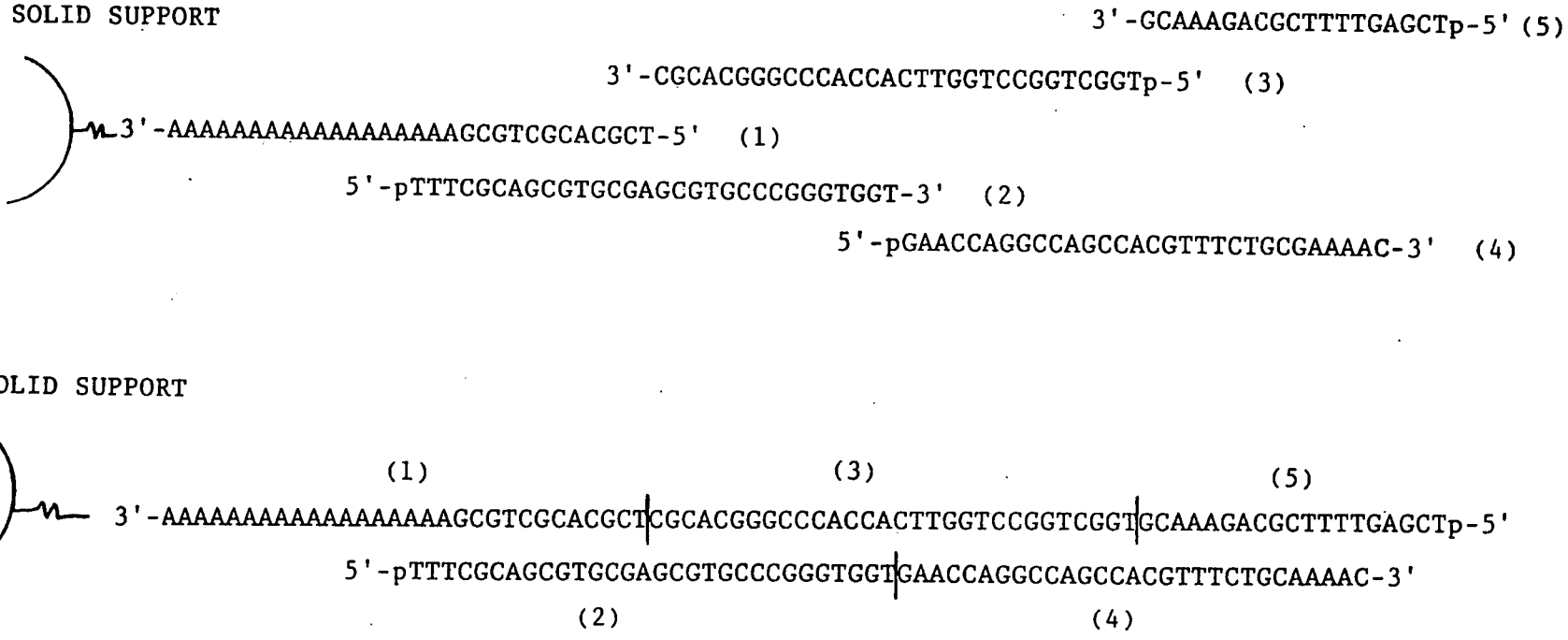

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02915

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 01/68; C12P 21/00; C12P 19/34; G01N 33/00; C07K 1/04
U.S.Cl.: 435/6; 435/68; 435/91; 436/86; 436/94; 530/334;
SEE ATTACHMENT

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/ 6, 68, 91, 172.3 436/ 86, 94 530/ 334 536/ 27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CHEMICAL ABSTRACT DATA BASE (CA):1967-1989; BIOSIS DATA BASE: 1969-1989;KEYWORDS: solid phase, gene, oligonucleotide, assembly, synthesis, protein, biopolymer, polypeptide, polysaccharide, muta?

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	NUCLEIC ACIDS RESEARCH, Volume 15, issued 1987, August, (HOSTOMSKY ET AL.), "Solid-Phase Assembly of Cow Colostrum Trypsin Inhibitor Gene", See pages 4849-4856 particularly the abstract.	1-7
Y		8-10 and 12-13
Y	ANNUAL REVIEW OF GENETICS, Volume 15, issued 1981, November, (D. SHORTLE), "Directed Mutagenesis", See pages 265-294 particularly pages 266, 270 and 271	8-10 and 12-13
X	JOURNAL OF ORGANIC CHEMISTRY, Volume 48, issued 1983, March, (NAKAGAWA ET AL.), "Polymer-Bound Oxime: Application to the Synthesis of a Peptide Model for Plasma Apolipoprotein A-I", See pages 678-685 particularly the abstract.	1-8 and 11
X	CHEMICAL ABSTRACTS, Volume 107, no. 21, issued 1987, November, (LYLE ET AL.), "Chemical Synthesis of Rat Atrial Natruiretic Factor by Fragment Assembly on a Solid Support", See page 222, column 1, the abstract no. 198913u, J. Org. Chem., 1987, 52(17), 3752-9 (Eng.).	1-8 and 11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 SEPTEMBER 1989	26 OCT 1989

International Searching Authority	Signature of Authorized Officer
ISA/US	Richard C. Peet RICHARD C. PEET

Form PCT/ISA/210 (second sheet) (Rev.11-87)

I. CLASSIFICATION AND SUBJECT MATTER (CONTINUED)

IPC(4): C12N 15/00; C07H 15/12

U.S.Cl.: 435/172.3; 536/27